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The Malignant Conversion Step of Mouse Skin Carcinogenesis

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Multiple benign squamous papillomas commonly precede the development of an occasional squamous cell carcinoma in mouse skin carcinogenesis. The incidence of carcinomas can be enhanced by treating papilloma-bearing mice with mutagens such as urethane, nitroquinoline-*N*-oxide, or cisplatinum. This observation suggests that a genetic change is required for malignant conversion. The malignant phenotype is characterized by a marked reduction in the transcription of specific epidermal differentiation markers, a pattern which is useful for the early diagnosis of malignant conversion. Cells expressing a benign phenotype can be obtained by introducing the v-*ras*^{Ha} oncogene into cultured epidermal cells by a replication-defective retrovirus. Alternatively, benign tumor cells can be cultured from papillomas induced by chemical carcinogens *in vivo* or from carcinogen-treated mouse epidermis. In all cases, the benign phenotype *in vitro* is characterized by an altered biological response to changes in extracellular calcium, an important determinant of the differentiation state of cultured normal keratinocytes. Transfection of cloned plasmid DNA into benign tumor cells has revealed that transforming constructs of the *fos* oncogene induce malignant conversion, whereas *myc* and adenovirus *E1A* oncogenes do not. The *fos* carcinomas do not express differentiation-specific epidermal markers and secrete proteases such as transin and urokinase, a set of characteristics previously noted for chemically induced skin carcinomas. Cultured normal epidermal cells, exposed to the v-*ras* and the v-*fos* oncogenes simultaneously, are malignantly transformed. Alone, the *fos* oncogene does not detectably alter the phenotype of normal keratinocytes. These studies indicate that a limited number of genes is involved in epidermal carcinogenesis.

Introduction

In chemically induced mouse skin carcinogenesis, epidermal cancers are commonly preceded by the appearance of multiple benign squamous papillomas. Operationally, at least three distinct steps have been defined in mouse skin carcinogenesis (*1*). The first stage, initiation, occurs rapidly, is irreversible, and is commonly caused by mutagens. A single amino acid substitution mutation in the codon 61 of the c-*ras*^{Ha} gene has been causally related to the initiation step (*2,3*). Furthermore, introduction of the v-*ras*^{Ha} gene into normal epidermal cells is sufficient to initiate benign tumor formation (*4,5*). Initiated epidermal cells are resistant to signals that induce terminal differentiation in normal cells (*6,7*).

The second stage of mouse skin carcinogenesis, promotion, results from repeated and frequent applications of promoting substances that are generally nonmutagens (*1*). Most tumor promoters work by changing tissue homeostasis, providing an environment conducive for the selective clonal outgrowth of initiated cells (*8,9*). Initiation and promotion results in the production of multiple benign squamous papillomas, each of which is a monoclonal expansion of a single initiated cell (*10*).

The majority of papillomas remain benign throughout the lifetime of the animal (*11*). The low rate of spontaneous malignant conversion can be accelerated and the frequency enhanced by exposure of papilloma-bearing animals to mutagenic initiating agents (*12–14*). The introduction of specific oncogenes into benign tumor cells can also cause malignant conversion (*15–17*). Together these results suggest that a second somatic mutation in a benign tumor cell is sufficient to cause malignant conversion.

Genetic Basis of Malignant Conversion

Cell lines that produce papillomas when grafted as part of a reconstituted skin have been established in culture from chemically induced tumors (*18*). Two of these lines, SP-1 and 308, were derived from Sencar and Balb/c mice, respectively, and contain an activated c-*ras*^{Ha} gene containing an A → T transversion in codon 61 (*2,18*). These cell lines were stably transfected with plasmid DNA containing either a rearranged murine

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plasmacytoma-derived c-*myc* (minus exon 1), adenovirus 5 *E1A*, FBJ v-*fos*, or a human c-*fos*/*FBJ* v-*fos* chimera using cotransfection with a neomycin-resistance gene contained in pSV$_2$*neo* to select for transformants (*16*). The *E1A* and *myc*-containing plasmids did not alter the benign phenotype. Both *fos* constructs caused malignant conversion in either cell line as defined by the squamous cell carcinoma histology of tumors from grafted cells and the development of carcinomas after SC injection into athymic nude mice. The carcinomas produced by the *fos* oncogenes were devoid of staining for mouse keratin 1 (K1) but were positive for keratin 14 (K14), a marker pattern previously seen in chemically induced carcinomas (*19*). Tumors from *E1A*, *myc*, or pSV$_2$*neo* transfectants express K1, although in a focal distribution, a pattern common to dysplastic papillomas (*19*). *fos*-Induced carcinomas were indistinguishable from parental benign tumor cells by several *in vitro* markers of transformation. The *fos* transfection did not alter expression of the mutant c-*ras*^{Ha} to cause malignant conversion.

Undocumented genetic changes from establishment of parental cells *in vitro* could have contributed to the complementary action of the *fos* oncogene in malignant conversion in the foregoing studies. Cooperativity among *ras* and *fos* oncogenes in carcinoma induction was therefore tested directly in primary epidermal cells. Three days after isolation and cultivation, newborn keratinocytes were co-infected with v-*ras*^{Ha} and v-*fos* retroviruses that were replication defective (*5,20*). Infected keratinocytes were removed from culture and tested for tumor formation *in vivo*. In eight independent experiments, combined exposure to v-*fos* and v-*ras*^{Ha} resulted in squamous cell carcinomas, while exposure to v-*ras*^{Ha} only produced squamous papillomas and v-*fos* only produced normal skin. The tumors evolving from combined infection with v-*fos* and v-*ras*^{Ha} expressed K14 but not K1. Nucleic acid hybridization studies of RNA isolated from tumors from all groups indicated that the v-*ras*^{Ha} oncogene was expressed in the papillomas and both the v-*ras*^{Ha} and v-*fos* oncogenes were expressed in carcinomas. These results support the conclusion that cooperation between a *fos* and *ras* oncogene is sufficient to produce squamous carcinomas of keratinocyte origin. Furthermore, activation of *fos* alone may yield a normal skin phenotype, although such cells could experience malignant conversion by activation of a single complementing *ras*^{Ha} oncogene.

Conclusions

Our studies indicate that a single gene is sufficient to convert benign skin tumors to malignancy. Under certain conditions the *fos* oncogene can cause this change. the *fos* oncogene may exert its converting action via transcriptional enhancement of specific cellular genes, in conjunction with API, a mammalian transcriptional activator (*21,22*). Among the genes regulated by *fos*/API are secreted proteases, such as transin and collagenase (*23*). The elaboration of proteases as an early event in malignant conversion is consistent with the phenotypic differences cataloged among benign and malignant squamous tumors (*19,24*). As initiated cells already express an intrinsic defect in their differentiation program by virtue of the initiating mutation, protease secretion could lead to a major disruption of the extracellular environment (e.g., stroma, basement membrane) required to maintain proper structural organization in the benign tumor and thus proper expression of differentiation markers. Loss of extrinsic components of tissue regulation could cause the dysplastic histotype observed during malignant progression. Dysplasia is associated with loss of specific markers when analyzed by molecular probes (*25*). The loss of extrinsic control of a normal regulatory program could also deregulate the positional control of proliferation and lead to additional genetic changes associated with malignant progression.

A number of inhibitors have been described for individual proteases within a protease cascade (*26*). Cystatin A, a specific inhibitor of thiol proteases, is commonly deficient in malignant, but not benign skin tumors (*27*). The loss of an inhibitor could result in an enhancement of proteolytic activity, having similar phenotypic consequences as enhanced expression of the protease. One implication of such reasoning is that protease inhibitors could comprise one class of tumor suppressor genes.

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